COMPARATIVE TOXIN PRODUCTION IN DIPHTHERIA STRAINS.*

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The diphtheria antitoxin made by the New York Health Department is all produced by means of the action of a single strain of diphtheria organisms known in the laboratory as No. 8.

The organism used was obtained originally in the year 1895 in the course of ordinary routine culture examinations, from the throat of a child who had so slight an attack of diphtheria that it was at first diagnosed as tonsilitis. The animal tests which were being made with many strains at that time in the search for a good toxin producer showed this strain to be more active than any of those tried, and it was therefore selected for use in the preparation of toxin for the inoculation of horses. It has been constantly in use since that time for this purpose, has been kept growing in neutral broth, transferred about every third day, and has shown a remarkable persistence in vigor and toxin production. While this has fluctuated within wide limits at times, yet the careful studies made by Dr. A. W. Williams soon after isolation,[1] and confirmed by all subsequent work with the strain, show this fluctuation to be due to some alteration in the culture medium rather than to any inherent change in the organism itself, for a more careful preparation of the broth is always followed by a return to the same high toxin production, 1/400 to 1/500 c.c. and occasionally as high as 1/800 to 1/1,000, proving fatal within four days to medium sized guinea-pigs.

Morphologically the organism has remained unchanged during this long period of growth in the laboratory except that the rods are somewhat longer than when first isolated. In agar it has always produced very characteristic spreading colonies, and grows in broth with a typical heavy pellicle lying above a clear fluid.

Many comparative tests in the laboratory have always failed to find a culture stronger than No. 8 in toxin production, or even another equally active, but because of Dr. Park's desire to be thus

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[1] *Jour. Med. Res.*, 1902, 8, p. 83.

provided for the important work of toxin production it was decided to make another attempt, taking a large number of strains from different sources, and testing for toxin production soon after isolation, and again after a long period of cultivation; these results to be compared with those given by No. 8 grown under exactly similar conditions.

For this purpose 100 diphtheria cultures were obtained without selection, 25 being taken from the throats of diphtheria patients in the Willard Parker Hospital, and 75 from the cultures sent for diagnosis from the city to the Health Department laboratories. In the private patients, membrane was stated to be present on the throat in 67 cases, not mentioned in seven cases, absent in one case. Membrane was present on the throats of all the hospital cases, and generally in considerable amount, as nearly all were cases of a severe type. All strains without exception showed typical diphtheria bacilli, that is to say, forms corresponding to Wesbrooke's types A, C, and D. In most strains these were the predominating types, and in many almost the only forms present. Such strains gave from the beginning a vigorous growth in broth, with typical pellicle formation, and, on agar, characteristic spreading colonies. The other strains showed, besides the typical organisms, many smaller, more evenly staining forms on Loeffler's blood serum, and in most cases these strains grew with difficulty at first in broth, with pellicle either slight or absent, and on agar showed somewhat small dense colonies with but little tendency toward spreading.[1]

All strains were carried on in neutral broth cultures at 37° C., transferred every third day, with frequent platings to insure purity. In those strains that were at first less characteristic, there was an increasing tendency in later cultures toward better growth, with pellicle production in broth and characteristic colony formation on agar, but in a few cases strains remained somewhat typical throughout in these media, although never failing to produce characteristic bacilli when grown on Loeffler's serum tubes.

After an interval long enough to allow the organisms to become accustomed to growth in an artificial medium, generally at about

[1] The individual organisms of these strains grown on agar were not studied, but they probably are strains producing large segmenting forms on agar as described by Williams.

the sixth to the 20th transfer, sometimes later, all strains were tested for toxin production.

Broth cultures grown in the incubator for eight days were examined by means of transfer to agar plates and blood-serum tubes, carbolic acid was added in 1 per cent amount, and after two days' interval subcutaneous inoculations were made into medium sized guinea-pigs. The dose ranged from 0.1 to 0.005 c.c., according to culture conditions and size of animals, while in a few of the less toxic strains as much as 3 c.c. was given in later tests. At the same time a corresponding test was made with the same quantity of a culture of No. 8 grown in the same broth, and under the same conditions.

The result showed that most of the strains, including some of those having the most characteristic cultural properties, produced much less toxin than the stock culture No. 8. Many failed to kill in less than 0.1 c.c. and a few were not fatal even in larger amounts; while death, when it did occur, was generally much later than in the control animals inoculated with No. 8. With a few strains in these early tests the results were at first more nearly parallel, guinea-pigs injected with 0.1 to 0.02 c.c. dying as soon as, and in one or two cases even a little earlier than, the corresponding control animal, but when successively smaller doses were given, in all cases there was a lengthening interval, the new strain always showing less toxicity than the stock culture.

All strains were now again carried on for some time in broth and after a varying number of transfers, generally from 30 to 40, and in those first isolated from 80 to 100, or more, the strains were again incubated for toxin production, together with No. 8, and tested as before.

The results were even more disappointing than in the earlier series as to finding a better culture than No. 8, or even a second equally active toxin producer. In some cases the amount of toxin production was comparatively unchanged, but the majority of the strains, among them some of those formerly giving the best results, now fell still farther behind No. 8.

The toxin from five strains failed to kill in less than 3 c.c. and that from nine others was not fatal even in this amount, but this result

was evidently largely due to the lack of ability in these organisms to grow well in the broth used, for when, at Dr. Williams' suggestion, they were cultivated for several transfers in ascitic growth and then tested for their virulence, in every case but one, death followed the inoculation of 0.1 c.c. of the living culture. This one was classed with the non-virulent and hence non-toxin producing organisms. The amount of toxin production in ascitic broth was not tested. Three other strains grew with such difficulty in ordinary broth that no satisfactory toxin tests could be made with the cultures, but grown in ascitic broth and tested for virulence, these strains also proved fully active in doses of 0.1 c.c. These results correspond exactly with those obtained by Dr. Williams in 1902. The best results in toxin production obtained with the entire series may be summarized as follows:

TABLE 1.

3	strains	killed	in	0.005	c.c.
11	"	"	"	0.01	"
25	"	"	"	0.02	"
6	"	"	"	0.033	"
11	"	"	"	0.05	"
27	"	"	"	0.1	"

The following table shows the relation between toxin production and virulence in the 17 strains producing little or no toxin in ordinary broth, and tested for virulence in ascitic broth.

TABLE 2.

TOXIN VIRULENCE.

	NEUTRAL BROTH		ASCITIC BROTH	
Strain	Dose	Result	Dose	Result
8	3 c.c.	Lived	0.1 c.c.	x 3 days
31	3 c.c.	x 2 days	0.1 c.c.	x 2 days
32	3 c.c.	Lived	0.1 c.c.	Lived
43	3 c.c.	Lived	0.1 c.c.	x 3 days
44	3 c.c.	Lived	0.1 c.c.	x 3 days
59	3 c.c.	x 2 days	0.1 c.c.	x 3 days
61	3 c.c.	Lived	0.1 c.c.	x 3 days
63	3 c.c.	x 2 days	0.1 c.c.	x 4 days
66*	.....	.....	0.1 c.c.	x 1 day
85	3 c.c.	x 2 days	0.1 c.c.	x 3 days
90*	.....	.....	0.1 c.c.	x 3 days
94	3 c.c.	Lived	0.1 c.c.	x 3 days
95	3 c.c.	Lived	0.1 c.c.	x 2 days
96	3 c.c.	Lived	0.1 c.c.	x 3 days
97	3 c.c.	x 2 days	0.1 c.c.	x 3 days
98	3 c.c.	Lived	0.1 c.c.	x 2 days
99*	.....	.....	0.1 c.c.	x 3 days

* No satisfactory toxin tests, owing to poor growth.

The 17 virulence tests with ascitic broth are given in detail contrasted with the toxin tests of the same strains in broth without ascitic fluid, as illustrative of the extreme sensitiveness of the diphtheria bacillus to slight changes in its nutrient medium. The overlooking of this fact is probably accountable for many of the conflicting statements in the reports of different investigators. Further work in this direction might have brought out finer comparative differences.

The result of this series of tests, carried on through so many transfers, and with so large a number of cultures, emphasizes strongly the unusual vigor and toxin production of the No. 8 laboratory strain, and the retention of these qualities for so long a time in an artificial medium is a striking illustration of the relative stability of the diphtheria organism.